Identification of Human Autologous Cytotoxic T-Lymphocyte-Defined Osteosarcoma Gene That Encodes a Transcriptional Regulator, Papillomavirus Binding Factor

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ABSTRACT

The prognosis for patients with osteosarcoma who do not respond to current chemotherapy protocols still remains poor. Toward the goal of establishing efficacious peptide-based immunotherapy for those patients, we previously developed an autologous pair of CTLs and an osteosarcoma cell line. In the current study, we screened the cDNA library of this osteosarcoma cell line using an autologous CTL clone and identified cDNA encoding an antigen. The isolated cDNA was identical to papillomavirus binding factor (PBF), which was recently reported as a DNA binding transcription factor cooperating with RUNX1. Reverse transcription-PCR analysis revealed that PBF was expressed in 16 of 19 cases of bone and soft-tissue sarcoma cell lines (5 of 6 of osteosarcoma lines) and 57 of 76 sarcoma tissue samples (11 of 14 of osteosarcoma tissues). Also, PBF was expressed in 10 of 13 epithelial cancer cell lines and 20 of 34 of cancer tissues. In contrast, PBF was detected in some normal organs including ovary, pancreas, spleen, and liver by reverse transcription-PCR but was restricted in the cytoplasm by immunostaining and undetectable by Western blotting. Furthermore, a 12-mer peptide, CTACRWKKACQR, located at the COOH terminus of PBF, was found to be a minimum requirement for recognition by the CTL clone in the context of the HLA-B*5502 molecule. These findings suggest that PBF is a shared tumor-associated antigen, which may serve as a source of peptides applicable to peptide-based immunotherapy for osteosarcoma and other malignant tumors.

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor of childhood and adolescence (1). Despite the advent of combined surgical and chemotherapeutic modalities, 30–40% of patients with osteosarcoma still succumb to metastatic diseases (2). For those patients, development of new therapeutic modalities is urgently needed.

Currently, there is a growing realization that active immunotherapy serves as the fourth therapeutic modality for malignant tumors following surgery, chemotherapy, and radiotherapy. The identification of tumor-associated antigens recognized by CTLs and subsequent clinical trials using peptide vaccine formulations derived from those antigens have shown tumor regression in patients with malignant melanoma and several carcinomas (3, 4). Meanwhile, no such antigens have yet been identified in osteosarcoma, even though CTLs reacting with autologous osteosarcoma cells were successfully developed by Slovin et al. (5) more than a decade ago. The reasons for this delay include the following: (a) relatively low immunogenicity of osteosarcoma because there are few examples of spontaneous tumor regression and tumor-infiltrating lymphocytes (6, 7); (b) the practical difficulty in establishing osteosarcoma cell lines and autologous CTLs (5, 8); and (c) the lack of suitable candidate genes for a reverse immunological approach such as a tumor-specific fusion gene (9, 10). An alternative proposal to use peptides derived from known shared antigens in antiosteosarcoma immunotherapy such as MAGE-3 and SART-1 (11, 12) requires preclinical evaluation of peptide antigenicity.

After attempts over a period of 3 years, we recently established an autologous tumor cell-CTL pair from a 16-year-old osteosarcoma patient (8). Using this pair in the present study, we carried out cDNA library expression cloning and identified an antigen and an epitope that sensitizes the antiauxologous osteosarcoma CTL clone.

MATERIALS AND METHODS

This study was approved under the institutional guidelines for the use of human subjects in research. Patients and/or their families gave informed consent for use of tissue specimens and blood samples in research.

Cell Lines and Transfectants. Cell lines used were osteosarcoma (OS2000, KIKU, Huo9, NY, HOS, and Saso2), Ewing’s sarcoma (A673, W-ES, NCR-EW2, SCCH196, SK-ES1, and RD-ES1), synovial sarcoma (HS-SY-II; 13); SW982 and Fuji (14), rhodomyosarcoma (HS729T, A204, and RD), fibrosarcoma (HT1080), lung adenocarcinoma (LHK-2 and KMG-A), head and neck squamous cell carcinoma (Fa-1, OSC20, OSC4, and HC-MA), esophageal carcinoma (KE-4), malignant melanoma (LG2-mel, LB3-mel, 888-mel, and 1102-mel), renal cell carcinoma (BB64-RCC), bladder carcinoma (LB905-BLC), and erythroleukemia (K562). EBV-infected B-cell lines (LGL-EB4 and OS2000-EBV) and 293EBNA cells were also used; OS2000, KIKU, and LHK-2 cell lines were established in our laboratory (8, 15). Other cell lines were kindly donated or purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan), Invitrogen Corp. (Carlsbad, CA), and American Type Culture Collection (Manassas, VA). HLA genotypes of osteosarcoma cell lines were as follows: (a) OS2000, A*2402, B*5502, B*4002, Cw*0102, Cw*0201, Cw*0301, Cw*0701, Cw*0801, Cw*1202; (b) KIKU, A*2402, A*0206, B*4006, B*5201, Cw*0102, Cw*1202; (c) Huo9, A*2401, A*3303, B*4403, B*5101, Cw*1402, Cw*1403; (d) NY, A*2601, B*5502, Cw*0303; and (e) HOS, A*0211, B*5201, Cw*1202.

HOS cells and 293EBNA cells were transfected with the expression vector pIRESpuro (BD Biosciences Clontech, Palo Alto, CA) encoding cDNA for HLA-B*5502, which had been subcloned from OS2000 cDNA. HOS transfectants were selected in RPMI 1640 containing puromycin (1 μg/ml) and 293EBNA transfectants were selected in DMEM containing neomycin (375 μg/ml) and puromycin (2 μg/ml). Stable transfectants were designated as HOS-B55 and 293EBNA-B55, respectively.

Antibodies. Monoclonal antibodies used were anti-HLA class I monoclonal antibody [mAb (W6/32; isotype IgG2a)] anti-HLA-A24 mAb (C7709A2.6; IgG2a) (16), and anti-HLA-B55 mAb [ME1; IgG1 as anti-HLA-B7-B27-Bw22 (B54, B55, and B56)]. These hybridomas were kindly donated or purchased from American Type Culture Collection.

Establishment of Autologous CTL Clones. Autologous CTLs derived from peripheral blood mononuclear cells against OS2000 cells (8) were plated at various dilutions in round-bottomed 96 microwells (Corning, Inc., Corning,
NY) in AIM-V supplemented with recombinant interleukin-2 (20 units/ml, a gift from Takeda Chemical Industries, Ltd., Osaka, Japan) and anti-CD3 antibody (40 ng/ml; BD Biosciences PharMingen, San Diego, CA). L929-EBV (cells (1 × 10^6 cells/well) and all irradiated and phospho-mononuclear cells (1.5 × 10^5 cells/well) were added as feeder cells. Cells were incubated at 37°C, and the medium was exchanged to AIM-V supplemented with 1000 units/ml recombinant interleukin-2 on day 10. CTL clones showing specific cytotoxicity against OS2000 cells in a standard 6-h 51Cr release assay were discarded, and 8 × 10^5 TcOScl-303 cells were added. After a 24-h incubation in AIM-V medium at 37°C, the amount of LDH in supernatant (100 μl) was measured by colorimetric assay using a LDH Cytoxidity Detection Kit (Takara, Ohtsu, Japan). OS2000 cells were used as a control target. Cytotoxicity was calculated using the following equation: LDH release = (sample release on incubation with CTL – spontaneous release on incubation with medium)/OS2000 release on incubation with CTL – spontaneous release on incubation with medium).

In blocking experiments, mAb (W6/32, C7709A2.6, or ME1) was added and incubated for 30 min at room temperature before coculture with TcOScl-303 cells. In experiments to determine the antigenicity of peptides, 293EBNA-B55 cells (8 × 10^5) were pulsed with 1 mg/ml peptide and DTT (200 μM) for 1 h at 37°C before incubation with TcOScl-303 cells.

cDNA Library Expression Cloning. A cDNA library was prepared from OS2000 mRNA using FastTrack 2.0 mRNA Isolation Kit (Invitrogen) and the Superscript Choice System (Invitrogen). The cDNA was ligated to HindIII-Nor adapters and digested by NorI. The resultant cDNA was cloned into the pCEPA vector (Invitrogen). Recombinant plasmids were electroporated into ELECTROMAX DH10B cells (Invitrogen) and selected with ampicillin (100 μg/ml). The library was divided into pools of 100–150 cDNA clones. Each pool was amplified, and plasmid DNA was extracted using a QIAprep spin miniprep kit (Qiagen, Hilden, Germany). 293EBNA-B55 cells (8 × 10^7) were transfected with 1.5 μg plasmid DNA using LipofectAMINE PLUS (Life Technologies, Inc.). The mixture was denatured at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, 57°C for 30 s, and 68°C for 1 min. Truncated cDNA variants and amino acid substitution variants (one codon was replaced with alanine- or serine-encoding codon) of PBF were generated by PCR and subcloned into pCEP4. Antigenicity of each synthetic peptide was evaluated by LDH release assay.

RESULTS

Cytotoxicity and HLA Restriction of the Autologous TcOScl-303 Clone. To attain the goal of establishing efficacious peptide immunotherapy for patients with osteosarcoma, we developed an autologous pair of CTLs and an osteosarcoma cell line, OS2000, from a 16-year-old patient. In the current study, we cloned those CTLs by limiting dilution and obtained TcOScl-301, TcOScl-303, and TcOScl-101. Of these three clones, TcOScl-303 showed the highest cytotoxicity against OS2000 cells and was therefore used in the subsequent experiments. As shown in Fig. 1A, the TcOScl-303 clone lysed OS2000 cells, but not 293EBNA or K562 cells, as determined by 6-h 51Cr release assays. In addition, cytotoxicity of TcOScl-303 against OS2000 cells was enhanced by pretreatment with IFN-γ. The phenotype of TcOScl-303, as determined by flow cytometry, was CD3+, CD4−, CD8+, and T-cell receptor positive (data not shown).

To determine HLA restriction, we evaluated the cytotoxicity of TcOScl-303 against allelogeneic osteosarcoma cell lines (Fig. 1B). As shown, TcOScl-303 selectively lysed OS2000 and NY cells, both of which express HLA-B*5502. To further confirm the HLA-B*5502 restriction of TcOScl-303 cytotoxicity, we generated HLA-B*5502-transfected HOS cells (HOS-B55). As shown in Fig. 1C, cytotoxicity of TcOScl-303 against HOS cells increased by exogenous expression of HLA-B*5502. These findings suggest that TcOScl-303 recognized putative shared antigens presented by the HLA-B*5502 molecule.

Cloning of cDNA Encoding an Osteosarcoma Antigen Recognized by the TcOScl-303 Clone. We then screened 1000 pools (1 × 10^6 cDNA clones) of the cDNA library constructed from OS2000 mRNA by a LDH assay using HLA-B*5502-transfected 293EBNA (293EBNA-B55) cells and the TcOScl-303 clone. Consequently, we identified four positive clones, all of which contained an identical cDNA fragment. Fig. 2A shows representative results of a positive clone, B9.1H4. TcOScl-303 clone selectively lysed 293EBNA-B55 cells transfected with B9.1H4 cDNA and the original OS2000 cells. Standard 51Cr release assays also confirmed the specific recognition of TcOScl-303 for B9.1H4 cDNA (Fig. 2B).

Structure of cDNA B9.1H4 Identical to PBF. The B9.1H4 clone contained 1908-bp cDNA, which was identical to the longest

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open reading frame of PBF (21) with deletion of 5′ sequence and additional 3′-untranslated region. There were no frameshifts or point mutations. Subsequently, we examined the antigenicity of PBF for TcOScl-303. As shown in Fig. 2A, TcOScl-303 selectively lysed 293EBNA-B55 cells transfected with cDNA for wild-type PBF or 1B9.1H4 cDNA, in addition to OS2000 cells. Furthermore, lysis by TcOScl-303 was blocked by anti-HLA-B*5502 mAb and anti-HLA-class I mAb, but not by anti-HLA-A24 mAb (Fig. 2B), suggesting HLA-B*5502 restriction in recognition of PBF by TcOScl-303.

Expression of PBF in Tumors and Normal Organs. We next determined the expression of PBF in various tumors and normal tissues by RT-PCR, immunohistochemical staining, and Western blotting. Table 1 summarizes the results of RT-PCR analysis in bone and soft-tissue sarcomas. As shown, PBF was detected in 16 of 19 (84%) bone and soft-tissue sarcoma cell lines and 57 of 76 (75%) sarcoma tissue samples. With regard to osteosarcoma, PBF was detectable in 5 of 6 cell lines and 11 of 14 biopsy specimens (Fig. 3A). Table 2 summarizes the expression of PBF in epithelial cancer cell lines and tissues, showing positive PCR in 10 of 13 (77%) and 20 of 34 (59%) cases, respectively. In normal adult organs (Fig. 3B), PBF was detectable in the pancreas and ovary at substantial reactivity; however, PBF expression was weaker in the prostate, heart, liver, spleen, thymus, and testis and faint or undetectable in other organs. None of the fetal normal organs showed significant PBF product, although the pancreas and ovary were not included in the analysis.

For immunohistochemical analysis and Western blotting, we...
Papillomavirus binding factor (PBF) was determined in 16 adult and 8 fetal normal tissues. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) housekeeping gene was used as a positive control.

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<table>
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*PBF, papillomavirus binding factor.

Identification of Epitope for TcOScl-303 Clone. Finally, we attempted to determine the epitope of PBF recognized by the TcOScl-303 CTL clone. A number of truncated variants of PBF were transfected into 293EBNA-B55 cells and tested for TcOScl-303 recognition by LDH release assay. As shown in Fig. 5A, TcOScl-303 lysed 293EBNA-B55 cells when they were transfected with PBF variants containing the COOH-terminal end at 1866 bp or longer. In contrast, cytotoxic activity clearly fell when the COOH-terminal end of PBF was truncated at 1863 bp.

To further assess the antigenicity of the COOH-terminal region (337–1866 bp) of PBF, we substituted one codon at the terminus of this region with Ala- or Ser-encoding codon and tested for TcOScl-303 recognition in 293EBNA-B55 cells. As shown in Fig. 5B, substitutions of Arg at position 510 and Gln at position 509 did not particularly affect recognition by TcOScl-303. In sharp contrast, substitution of Cys at position 508 apparently reduced the amount of LDH release. A similar apparent reduction in LDH release was observed by substitutions of Cys at position 502 and Ser at position 501.

On these findings, we tested the antigenicity of various synthetic peptides for the TcOScl-303 clone, focusing on the COOH-terminal end of PBF (501–510 amino acids). Of these, the 12-mer peptide CTACRWKKACQR (499–510 amino acids) was the most immunogenic for TcOScl-303 in the context of HLA-B*5502 (Fig. 5C).

DISCUSSION

In the present study, we identified PBF as an osteosarcoma antigen recognized by autologous CTLs using the cDNA expression cloning procedure. We found that a 12-mer peptide, CTACRWKKACQR, located at the COOH terminus of PBF was a minimum requirement for recognition by TcOScl-303 in the context of the HLA-B*5502 molecule. These findings suggest that PBF may serve as a source of therapeutic peptides that are effective in further improving survival rates of patients with osteosarcoma, especially those who are unresponsive to current chemotherapy protocols.

Tumor-associated antigen recognized by CTLs is the principal prerequisite for the development of antigen-specific cancer immunotherapy. However, given the technical difficulty in establishing autologous tumor-CTL pairs, antigen identification based on autologous pairs has come mostly from melanoma and renal cell carcinoma. In other tumors, including bone and soft-tissue sarcomas, a reverse immunology approach has been used and has defined antigenic peptides from tumor-specific fusion genes such as SYT-SSX in synovial sarcoma (9, 10), EWS-FLI1 in Ewing’s sarcoma (22), and PAX3-FKHR in alveolar rhabdomyosarcoma (10, 22). Because the peptides defined by the reverse approach are not always processed in tumor cells, those synthetic peptides need rigorous evaluation before clinical application. In addition, their therapeutic efficacy may not be as
promising as those defined by the autologous pair-based approach. In fact, vaccination trials of junction peptides from BCR-ABL [12 patients with chronic myelogenous leukemia (23)], EWS-FLI1 [12 patients with Ewing’s sarcoma (22)], and PAX3-FKHR [four patients with alveolar rhabdomyosarcoma (22)] only resulted in tumor remission in a single patient with Ewing’s sarcoma.

We used the LDH release assay to screen the cDNA library, even though this method is not as common as cytokine release assays. In our hands, none of cytokine release assays (IFN-γ, TNF-α, and GM-CSF) successfully detected the specific CTL responses against OS2000 cells due to spontaneous cytokine release from the TcOSc1-303 CTL clone. The value and reproducibility of LDH release assay have been confirmed by 51Cr release assays in the present study and have also been confirmed by other investigators (24, 25).

The clone isolated from a cDNA expression cloning procedure was identical to PBF, a newly defined transcription factor (21). PBF has been shown to regulate E2-mediated repression of genomic DNA of human papillomavirus type 8 in cooperation with RUNX1. The role of PBF in bone and soft-tissue sarcomas remains uncertain. We can only speculate that the functional cooperation between PBF and the RUNX family members (RUNX1, RUNX2, and RUNX3) may also exist in sarcomas because these members have been shown to play pivotal roles during normal development and in oncogenesis (26, 27). Notably, RUNX1 has been shown to be involved in the differentiation of osteochondroprogenitor cells (28), and RUNX2 (also known as CBFA1) functions as a master regulatory gene for osteoblastogenesis.

The peptide defined as the CTL epitope was unexpectedly a 12-mer form and had to be of a high concentration (1 mg/ml) for optimal sensitization. Such long antigenic peptides have been reported in the literature (29–35). Without peptide elusion or structural analysis, we are not able to comment on whether the 12-mer peptide is identical to...
the natural ligand presented by HLA-B*5502 on OS2000 cells or whether this peptide binds to the HLA with a structural modification such as central bulging or overhanging. The high peptide concentration requirement may be explained by multiple cysteine residues contained in the peptide, which cause formation of disulfide bonds in culture medium and interfere with HLA-peptide binding (36). The low affinity of the peptide to the HLA-B*5502 molecule may be another possible reason for this requirement.

Wide expression of PBF in osteosarcomas and other sarcomas encourages the use of PBF-derived peptides in antisarcoma immunotherapy. In this regard, it remains to be determined whether PBF defined in tumors by RT-PCR and immunohistochemistry truly serves as an antigen. As we determined previously for other antigenic peptides (9, 18), CTL induction and tetramer analysis on peripheral blood mononuclear cells from patients with PBF-positive tumors are prerequisites for clinical use of PBF-derived peptides. Also, it is informative to investigate the impact of PBF expression on clinical behaviors of the tumor. Because PBF was detected in several normal tissues including pancreas and liver by RT-PCR analysis, potential autoimmunity against normal tissues may also be a concern. This needs to be evaluated preclinically by peptide immunization of HLA-transgenic mice. However, localization in the cytoplasm rather than the nucleus and the relatively weak expression of PBF in normal tissues appear to diminish such a likelihood. It should be noted that clinical studies of peptides derived from other ubiquitous antigenic proteins [i.e., WT1 (37) and HER-2/neu (38)] have thus far reported no adverse effects related to autoimmunity.

Given the low prevalence of the HLA-B*5502 allele, the 12-mer antigenic peptide defined in this study is not widely clinically applicable. It is suitable to identify PBF-derived peptides that have high
affinity to HLA-A2 and HLA-A24, common alleles in both Japanese and Caucasians. Our experience in an ongoing Phase I trial of SYT-SSX-derived peptide for HLA-A24-positive synovial sarcoma patients should benefit subsequent clinical applications of PBF-derived peptides. Those peptides with affinity to HLA-A2 and HLA-A24 may be applied as vaccine (s.c. injection) in patients with corresponding HLA and PBF-positive tumors.

In conclusion, this is the first report of identification of an antigenic peptide derived from osteosarcoma. PBF proven as an osteosarcoma antigen may serve as a source of peptides that are available for immunotherapy not only for osteosarcoma but also for other sarcomas. The current study has gone a long way toward proving that tumor-associated antigens are identifiable by the autologous pair-based approach from relatively low immunogenic and similarly low prevalence tumors. These above-stated identifications should further the development of efficacious immunotherapy for a diverse range of malignant tumors.

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